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Effects of osmolality on inner mitochondrial transmembrane potential and ATP content in spermatozoa recovered from the testes of striped bass (Morone saxatilis)☆

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Abstract

The objective was to determine the effects of osmolality on the energy status of testicular spermatozoa of striped bass incubated in a TRIS free base-NaCl medium (pH 8) adjusted to either 300 (T300) or 600 mOsm/kg (T600) with NaCl. High mitochondrial inner transmembrane potential ($\Delta\Psi_m$) was assessed (flow cytometry) with the mitochondrial probe 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl- carbocyanine iodide (JC-1) and ATP was measured with a luciferin–luciferase assay. Spermatozoa maintained on ice were equally viable (>95% for T300 and T600) for up to 80 min, whereas sperm viability in artificial fresh water (FW) at 27 mOsm/kg decreased ($P < 0.05$) to 67% after 5 min, with only 3.5% viability at 25 min. After 20 min of staining, more spermatozoa ($P < 0.05$) maintained a high $\Delta\Psi_m$ in T300 than in T600 (80 and 50%, respectively). Sperm JC-1 aggregate (J_{agg}) fluorescence intensity was also greater ($P < 0.05$) in T300 than in T600 (10 and 5 channel number). The J_{agg} fluorescence was a function of oxidative phosphorylation; the percentage of cells containing J_{agg} fluorescence decreased to 3% in the presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an uncoupler of cell respiration and oxidative phosphorylation. After incubation for 30 min in the absence of CCCP, sperm ATP concentration was greater ($P < 0.05$) in T300 than in T600 (2.0 vs. 0.2 pmol/10^6 cells), but was below detectability in the presence of CCCP in either medium. In conclusion, we developed a unique approach to assess the energetic status of striped bass spermatozoa during storage and after activation, and concluded that the effects of osmolality must be considered in the design of activating and storage extenders to maintain striped bass sperm motility, viability, and fertility in vitro.

Keywords: Flow cytometry; JC-1; Carbonyl cyanide 3-chlorophenylhydrazone; Spermatozoa motility

1. Introduction

The spermatozoa of striped bass (*Morone saxatilis*), an anadromous species, remain quiescent in seminal plasma and become motile after spawning into fresh water, a process known as activation. Osmolality was a major regulator of motility in striped bass spermatozoa in vitro [1]. Striped bass spermatozoa were activated equally well in distilled water or in solutions of NaCl

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and KCl ranging from 100 to 300 mOsm/kg; however, at osmolalities >300 mOsm/kg, activation efficiency decreased as osmolality increased, with no activation induced when NaCl or KCl solutions were used to increase osmolality to 600 mOsm/kg. Approximately 90% of striped bass spermatozoa from fresh semen are activated in distilled water, but the duration of motility is only 30–60 s [1,2]. Striped bass spermatozoa are often held in media with 600 mOsm/kg, to delay activation for a few hours so that they can be used in laboratory experiments, or to maintain their fertility for cryopreservation [1]. Although motility and viability of spermatozoa of mammalian and avian species exhibited a negative reaction to osmotic stress [3], the effects of hypo- and hyper-osmotic conditions on viability of striped bass spermatozoa have not been determined.

Rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) spermatozoa had rapid loss of motility by 30 s after activation [4–6]. The decrease in flagellar beat frequency and percent motility in these species were associated with a progressive decline in ATP content [6,7]. It was concluded that oxidative phosphorylation could not replenish ATP fast enough to maintain motility in intact sperm of these species. Knowledge of striped bass sperm energy status before and after storage is lacking, but is of critical importance to design new extenders to prolong sperm motility, improve fertilization capacity, and decrease the negative effects of liquid storage and cryopreservation. In the present study, we investigated the effects of osmolality (300 and 600 mOsm/kg) on testicular sperm viability and energetics using two TRIS free base-NaCl media. Testicular spermatozoa were used to avoid urine contamination of manually expressed sperm. Sperm energy status was monitored by determination of changes in mitochondrial inner transmembrane potential ($\Delta\Psi_m$) [8] and measurement of ATP [9].

2. Materials and methods

2.1. Collection of spermatozoa

Mature (3 y) striped bass males were randomly selected from a population maintained under controlled photothermal conditions in the 40,000-L tank wet lab at the University of Maryland's Crane Aquaculture Facility [2]. Animal care and husbandry, as well as the collection of tissues, were conducted under an approved Institutional Animal Care and Use Committee Experimental Protocol (R-07-35). Water temperature and photoperiod were computer-controlled throughout the 2006–2007 gametogenic cycle to mimic average conditions for the Maryland reach of the Chesapeake Bay. In the spring, experimental males ($n = 40$) were moved into a 6400-L circular tank, part of the recirculating water system, and held at 15 ± 0.5 °C for the remainder of the 5-wk study. To stimulate spermiogenesis, each fish was given a cholesterol cellulose implant [10] containing 150 μg of mammalian gonadotropin-releasing hormone (Sigma Chemical Co., St. Louis, MO, USA) inserted into the dorsal lymphatic sinus, as previously described for striped bass [11,12]. Fish were euthanized in a 200 mg/L bath of tricaine methanesulfonate (Finquel®, Argent Chemical Laboratories, Redmond, WA, USA) buffered with sodium bicarbonate. Each lobe of the whole testes was carefully removed by first cutting the dorsal mesorchia and the anterior gonadal arteries before cutting the gonad just anterior to the urogenital bladder. The testes were gently dried with sterile absorbent cloths and then semen from each lobe's central lumen was squeezed (anterior to posterior) into 50 mL sterile conical tissue tubes. Sperm cells were held undiluted over ice and transported (15 min) to the USDA Animal Biosciences and Biotechnology Laboratory.

Sperm concentration was determined using a hemocytometer and only samples exhibiting ≥90% motility were used. Activation of motility was detected in a Makler counting chamber (TS Scientific Inc., Perkasie, PA, USA) using a Zeiss model D-7082 compound microscope (Berlin, Germany) at 400×. The percentage of motile spermatozoa was determined by counting spermatozoa (approximately 200–800 cells), then dividing the number of motile spermatozoa by the total number of sperm cells in the field of view [1]. Semen samples were held over ice and were not diluted until the start of each experiment.

2.2. Determination of viability

Sperm plasma membrane integrity (viability) was determined by flow cytometric analysis [13] of SYBR-14 and propidium iodide (PI) (Sperm Viability kit L-7011, Molecular Probes, Eugene, OR, USA). The stock solution SYBR-14 (1 mM in DMSO) was diluted to a 0.177 mM working solution in DMSO and 0.75 μL was added to each 1 mL of sample of $30–40 \times 10^6$ cells to a final concentration of 0.013 μM (1:1333 dilution of DMSO). The PI (2.4 mM in water) was added in 6 μL to each sample to a final concentration of 14.4 μM. Samples were analyzed on an Epics XL flow Cytometer (Beckman-Coulter Co., Hialeah, FL, USA) equipped with an argon laser operated at a wavelength of 488 nm and System II software to gate on 10,000 single

spermatozoa by forward and right-angle light scatter [8]. Cells positive for SYBR-14 and negative for PI were classified as viable.

2.3. Determination of spermatozoa with high $\Delta\Psi_m$

Semen samples and a set of 12 mm × 75 mm polypropylene assay tubes were held over ice during the procedure. The test tubes were prepared to contain 1 mL of 20 mM TRIS free base (#1503, Sigma–Aldrich, St. Louis, MO, USA) adjusted to pH 8.0 with HCl and either adjusted to 300 or 600 mOsm/kg with NaCl. Then, 1 μL of PI (2.4 mM in water) was added to each tube, for a final concentration of 2.4 μM. Less than 1 μL of semen was added to each tube to yield a final sperm concentration of approximately 40×10^6 sperm/mL. As a negative control for the experiment, one set of tubes were pre-loaded with 4 μL (5 mM in DMSO) carbonyl cyanide 3-chlorophenylhydrazone (CCCP; #C2759, Sigma–Aldrich), with a final concentration 20 μM upon addition of sperm. Then, 0.5 μL of the mitochondrial probe 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) stock solution (0.6 mM in DMSO) was added to each tube (final concentration, 0.3 μM) and incubated for 20 min prior to flow cytometry.

The percentage of viable spermatozoa with a high $\Delta\Psi_m$ (>80 to 100 mV) and their fluorescence intensity was determined flow cytometrically using JC-1 along with PI to identify and electronically gate-out dead cells [8]. The probe JC-1 is freely permeable to cells and undergoes reversible transformation from a monomer to an aggregate form (J_{agg}) when it binds to membranes having a high $\Delta\Psi_m$ [14]. The J_{agg} form is distinguished from the monomeric form by its bright 590 nm (red-orange) fluorescence in response to 488 nm excitation, whereas the monomer has 525 nm background ''green'' fluorescence in response to the same excitation. The standard filter configuration of the Beckman–Coulter Epics XL flow cytometer was altered for analysis of J_{agg} fluorescence, as described previously [8]. Data were recorded as percent of cells with a high $\Delta\Psi_m$ (J_{agg} fluorescence) and J_{agg} fluorescence intensity in channel number (CN) on a log scale.

2.4. Analysis of sperm ATP content

Sperm ATP concentration was determined as described previously [9]. Briefly, a 100 μL aliquot of each sample (40×10^6/mL) was collected after completion of the in vitro treatments and incubated with 1 μL of a 100× phosphatase inhibitor solution (#P5728, Sigma–Aldrich) for 30 min after the experiment (to inhibit ATP degradation) and was stored at −20 °C until extraction of ATP. Boiling released ATP which was quantified using a luciferin–luciferase assay adapted to measurement of bioluminescence on a SpectraFluor Plus plate reader (Tecan Group Ltd., Maennedorf, Switzerland) and recorded as pmol of ATP/10^6 sperm.

2.5. Experiment 1: Effect of osmolarity on sperm viability

Testicular spermatozoa collected from six males were used within 1 h of recovery to determine the effects of medium osmolality on sperm viability. The sperm sample from each fish was divided into three portions and diluted to 80×10^6/mL in artificial fresh water (FW), composed of 1 mM NaCl, 0.4 mM KCl, 0.5 mM $CaCl_2$, 0.05 mM $MgSO_4$, 27 mOsm/kg and pH 7.6 [15] or suspended in 20 mM TRIS base-NaCl (adjusted to pH 8 with HCl), and adjusted to either 300 (T300) or 600 mOsm/kg (T600) with NaCl. Samples were incubated over ice and aliquots were removed after 1, 5, 25, and 80 min of incubation to stain with SYBR-14 and PI for flow cytometry. The experimental design was a randomized complete block with fish as the blocking variable, the three medium types as a fixed effect, and time of incubation as a repeated measure.

2.6. Experiment 2: Effects of osmolality on $\Delta\Psi_m$ and sperm ATP

Testicular spermatozoa from six different males were used within 1 h of recovery to determine the effects of medium osmolality on sperm energetics. The sperm sample from each fish was divided into two portions and diluted to 80×10^6/mL in T300 or T600. The FW treatment was not included in this experiment, because sperm plasma membranes were compromised within 5 min of addition of FW in experiment 1. Spermatozoa in T300 and T600 received 0 or 20 μM of CCCP. Then aliquots were removed to load with JC-1 and PI for flow cytometry. The experimental design was a randomized complete block, with fish as the blocking variable with a 2 × 2 factorial arrangement of TRIS medium osmolality and CCCP concentration as fixed effects.

Samples of spermatozoa from the same fish were also used for ATP determination. After dilution with T300 or T600, 100 μL aliquots were removed immediately for 30 min phosphodiesterase inhibitor treatment and frozen. Additional samples of spermatozoa diluted with T300 or T600 received 0 or 20 μM of CCCP and were incubated over ice for 20 min (to

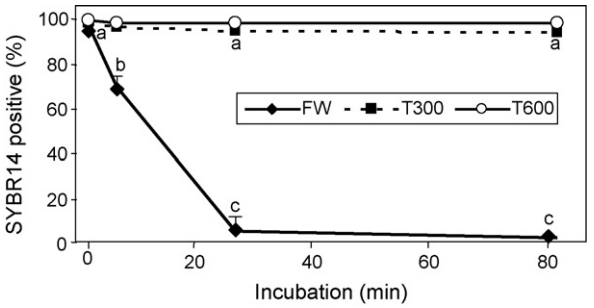

Fig. 1. Effect of extender osmolality on viability of testicular striped bass sperm ($n = 6$ fish) stained with SYBR-14/PI and analyzed by flow cytometry. Extenders were artificial fresh water (FW), pH 7.6 and 27 mOsm/kg and TRIS-free base-NaCl, 20 mM Tris base pH 8, at either 300 mOsm/kg (T300) or 600 mOsm/kg (T600). No significant difference between T300 and T600. [a–c]Within a line, means without a common superscript differed ($P < 0.05$).

correspond to the 20 min incubation for JC-1 staining time) and 100 mL aliquots were removed for 30 min phosphodiesterase inhibitor treatment and frozen. The experimental design was a randomized complete block, with fish as the blocking variable and the six treatment combinations as a fixed effect.

2.7. Statistical analysis

Data were analyzed using the mixed model analysis of variance procedure [16] in the Statistical Analysis System software, Release 8.02 (SAS Institute Inc., Cary, NC, USA) as described for each experiment. Model diagnostics included testing for a normal distribution of error residuals and for homogeneity of treatment variance [16]. Comparison of least squares means was made by the LSMEANS statement using the DIFF option.

3. Results

3.1. Experiment 1

Changes in viability with time of incubation of testicular spermatozoa extended in FW, T300, or T600 are shown (Fig. 1). Spermatozoa extended in T300 and T600 were viable (>95%) throughout the 80 min incubation period. In contrast, viability of spermatozoa suspended in FW decreased ($P < 0.05$) from 95% at 1 min to 67% at 5 min of incubation, with only 3.0 and 0.3% viable at 25 and 80 min, respectively.

3.2. Experiment 2

The effects of extender osmolality and CCCP concentration on sperm mitochondrial energetics in terms of the changes in the percentage of spermatozoa with a high $\Delta\Psi_m$ and J_{agg} fluorescence intensity are shown (Table 1). Spermatozoa held for 20 min in T300 were highly energized, with 80% having a high $\Delta\Psi_m$, above the threshold for J_{agg} fluorescence. In contrast, the percentage of spermatozoa with high $\Delta\Psi_m$ after being held in T600 for 20 min was decreased ($P < 0.05$) to 50% compared to T300. The addition of CCCP before the 20 min incubation in T300 and T600 reduced ($P < 0.05$) the percentage of spermatozoa having high $\Delta\Psi_m$ to 4 and 1%, respectively. The magnitude of J_{agg} fluorescence of spermatozoa incubated in T300 was 2-fold greater ($P < 0.05$) than for those incubated in T600. The presence of CCCP during the incubation decreased ($P < 0.05$) the J_{agg} fluorescence intensity from 10 and 4.5 CN, respectively, in T300 and T600 without CCCP to 1.2 CN in both T300 and T600 with CCCP.

The effects of extender osmolality and CCCP concentration on sperm ATP concentration are shown (Table 2). The ATP content of spermatozoa after 30 min treatment with phosphatase inhibiter in T600 was less than that of T300 (0.77 vs. 2.9 pmol/10^6 cells, $P < 0.05$). The additional 20 min incubation (50 vs. 30 min) further reduced ATP ($P < 0.05$) and maintained the difference between T300 and T600. The presence of CCCP reduced ATP to essentially non-detectable levels.

Table 1
Mean (±S.E.M.) effects of TRIS-free base-NaCl extender osmolality, 300 (T300) and 600 mOsm/kg (T600), and an uncoupler of electron transport and oxidative phosphorylation, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), on mitochondrial inner transmembrane potential in striped bass sperm ($n = 6$ males)

Medium	CCCP (μM)	Viable sperm with a high $\Delta\Psi_m$ (%)	Magnitude of J_{agg} fluorescence (channel number)
T300	0	79.9 ± 1.2^a	10.07 ± 1.15^a
	20	3.8 ± 0.1^c	1.27 ± 0.06^c
T600	0	49.6 ± 0.5^b	4.48 ± 0.52^b
	20	1.2 ± 0.1^c	1.18 ± 0.05^c

Within a column, means without a common superscript (a–c) differed ($P < 0.05$).

Table 2
Mean (±S.E.M.) effects of TRIS-free base-NaCl extender osmolality, 300 (T300) and 600 mOsm/kg (T600), and an uncoupler of electron transport and oxidative phosphorylation, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), on ATP concentration in striped bass sperm ($n = 6$ fish)

Medium	CCCP (μM)	Incubation time (min)	ATP (pmol/10^6 cells)
T300	0	30[1]	2.94 ± 1.61^{a}
	0	20 + 30[2]	2.12 ± 0.69^{b}
	20	20 + 30	0.11 ± 0.02^{cd}
T600	0	30	0.77 ± 0.11^{c}
	0	20 + 30	0.21 ± 0.07^{cd}
	20	20 + 30	0 ± 0^{d}

Within a column, means without a common superscript (a–d) differed ($P < 0.05$). [1]Immediate 30 min incubation with phosphatase inhibitor at 24 °C. [2]Incubation over ice for 20 min, followed by incubation with phosphatase inhibitor (at 24 °C) for 30 min.

4. Discussion

Activation of motility in striped bass spermatozoa was accomplished by dilution in water or in media that were 300 mOsm/kg or less, whereas suspension in media of 600 mOsm/kg completely blocked activation [1]. In contrast to the known short duration of motility after activation, in the present study, viability was maintained at nearly 100% in spermatozoa held in T300 and T600 media for up to 80 min. A new finding from our current study was that a common method to activate striped bass sperm, FW (or distilled water, Guthrie unpublished), decreased viability within 5 min and nearly all cells were dead after 25 min. Thus, with respect to the fertility of striped bass spermatozoa in vitro, it will be important to consider the effects of media osmolality on the fertilizing capacity of striped bass spermatozoa used for in vitro fertilization (incubating spermatozoa and oocytes in water for 5–10 min [2]).

In spite of the rapid loss of motility, most (80%) of spermatozoa were energized (high $\Delta\Psi_m$) when incubated in T300 over ice for 20 min. However, compared to T300, 50% fewer cells were energized when incubated in T600. In addition, the J_{agg} fluorescence intensity for individual energized spermatozoa held in T300 was two-fold greater than those held in T600. Changes in sperm ATP content followed a pattern similar to that for the percentage of spermatozoa cells with a high $\Delta\Psi_m$ and their J_{agg} fluorescence intensity. The sperm ATP content was greater in spermatozoa incubated in T300 than T600 (2.9 vs. 0.8 pmol/10^6 cells). Thus, we demonstrated an energetic 'cost' associated with the using solutions of high osmolality (600 mOsm/kg), which justifies the search for a better extender for hypothermic liquid storage of striped bass sperm.

The coupling of electron transport to oxidative phosphorylation maintains a high $\Delta\Psi_m$ required for mitochondrial ATP production in somatic cells [17]. Even though striped bass spermatozoa were no longer motile in T300 and never motile in T600, electron transport was still coupled to oxidative phosphorylation after the 20 min incubation period. Treatment of spermatozoa with the uncoupling agent CCCP reduced the number of spermatozoa capable of maintaining a high $\Delta\Psi_m$ and dramatically inhibited ATP production. Based on other studies of rainbow trout and carp spermatozoa, changes in cellular ATP content were closely linked to motility activation and the duration of motility [5,6,7]. In comparison with intact spermatozoa with motility duration of 30 s, demembranated rainbow trout spermatozoa maintained motility for up to 20 min in the presence of mM concentrations of ATP and μM concentrations of cAMP or ADP alone or in combination with cAMP [6]. In contrast to demembranated rainbow trout sperm, the duration of motility in demembranated striped bass spermatozoa incubated with ATP was low, only 15–30 s [1], indicating that the rapid loss of motility in intact striped bass spermatozoa our study could be independent of the presence of ATP. Perhaps, in addition to a potential decline in ATP production after activation in striped bass sperm, ATP transport and utilization were negatively affected.

In conclusion, we developed a unique approach to assess the energetic status of striped bass spermatozoa during storage and after activation. Furthermore, we concluded that the effects of osmolality must be considered in the design and use of activating and storage/freezing solutions to maintain the motility, viability, and fertility of striped bass spermatozoa in vitro.

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